ACTION OF RENNIN AND PEPSIN ON β-CASEIN: INSOLUBLE AND SOLUBLE PRODUCTS

SUMMARY

β-Casein was hydrolyzed to a considerable extent by the enzymes pepsin and rennin at pH 6.4. Pepsin hydrolyzed β-casein more extensively than rennin when given sufficient time. Hydrolysis of β-casein was evidenced by an increase in solubility at pH 4.7, and an increase in solubility in 2% trichloroacetic acid (TCA). Solubility in 12% TCA was about the same before and after enzyme treatment; hence, it was not a suitable reagent for demonstrating hydrolysis. The insoluble portion (pH 4.7; 2% TCA) of β-casein after enzyme treatment was heterogeneous, containing at least three components. The soluble fraction was very heterogeneous and contained ten or more components. The major products released by rennin and pepsin have similar electrophoretic behavior, but there were differences among the minor products.

Reports on the action of the enzymes rennin and pepsin on β-casein have been diverse. Some have concluded that only α-casein is altered by rennin, whereas β-casein and γ-casein are not attacked (3, 1), but are merely coprecipitated unchanged along with the altered α-casein in the presence of calcium salts (3). Others have reported that β-casein shows only a slow general proteolysis (6, 7); that the prolonged action of rennin on β-casein gives a product no longer precipitable with calcium ion (9); and that β-casein is split into two components in 4 hr. by rennin (8). A recent report has shown that β-casein is solubilized considerably by pepsin in 5 min. (10).

The present investigation was undertaken to learn more about the action of the enzymes rennin and pepsin on β-casein, and to clarify the reports just cited. Since some of the conflict appeared to be due to the choice of conditions for precipitating the treated β-casein, precipitation at pH 4.7 as well as precipitation with two concentrations (2 and 12%) of trichloroacetic acid (TCA) were compared. The components of the insoluble and soluble fractions were investigated at the same time by electrophoresis and chromatography.

EXPERIMENTAL PROCEDURE

Enzymes. Pepsin was a crystalline, commercial product. Rennin was a highly purified product obtained through the courtesy of Dr. R. A. Sullivan, National Dairy Research Laboratories, L. I., N. Y. This rennin had the same specific activity as crystalline rennin.

β-Casein was prepared by the method of Hipp *et al.* (5), utilizing differential solubility in aqueous urea solutions.

Preparation of dry fractions. β-Casein and all precipitates were dried by

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washing with acetone and ether and removing the solvent in a stream of air. Solutions were concentrated and dried by a rapid, large-surface vacuum technique (4) at 30-35° C.

It was observed that all washings in all experiments, after evaporation of ether, left some yellowish, fat-like residue. These residues were not analyzed further.

Enzyme reaction. A 2% β-casein solution was prepared by dissolving 5 g. of dry protein in a mixture of 2.5 ml. of 1 *N* NaOH and 240 ml. of distilled water, and the solution was adjusted to pH 6.4. Five milligrams of enzyme was dissolved in 5 ml. of water and added to the casein solution at 30° C. The reaction was continued for a definite time at 30° C. The enzyme reaction was stopped either with TCA or heat, according to the type of experiment being run.

(a) *pH 4.7 precipitation.* The enzyme reaction was stopped by placing the flask in a boiling water bath and stirring for 15 min. The contents of the flask were quickly cooled to room temperature, adjusted to pH 4.7 with 0.1 *N* HCl, and centrifuged. Nitrogen and phosphorus were determined in the supernatant solution. The precipitate was dried as described above. The pH 4.7–soluble portion was dried by a vacuum technique and was fractionated with TCA as described for TCA precipitation. Three fractions were obtained: 2% TCA–insoluble, 12% TCA–insoluble, and 12% TCA–soluble.

(b) *TCA precipitation.* Twenty-five per cent TCA solution was added to a solution of the enzyme reaction products until a concentration of 2% was reached; the mixture was then centrifuged, and nitrogen and phosphorus were determined in the supernatant solution. The supernatant was extracted with ether six times to remove the TCA; then the aqueous phase was adjusted to pH 7.0 with NaOH and evaporated to dryness by a rapid vacuum technique (2). The portion of the β-casein insoluble in 2% TCA was dissolved in dilute NaOH and then reprecipitated at pH 4.7 with HCl. The supernatant of the pH 4.7 precipitation was dialyzed against water and dried by the rapid vacuum technique.

Similarly, 50% TCA was added to another portion of the enzyme reaction products until a concentration of 12% was reached. Subsequent steps were the same as those for the 2% TCA precipitation.

Analytical methods. The determination of nitrogen and phosphorus has been described previously (10).

Electrophoresis was carried out with a 1% solution at 1° C. in a Tiselius type electrophoresis apparatus for 3 hr. with a 11-ml. volume, standard, full-length cell. All buffer solutions were of ionic strength 0.1, with 80% contributed by NaCl. The buffering salts were: pH 8.6–veronal, pH 6.7–phosphate, pH 5.6–acetate.

Paper electrophoretic analyses of proteins and peptides were performed in a Durrum type electrophoresis cell (Spinco Model R).[2] Electrophoresis was done at 5° for 16 hr. in a veronal buffer, pH 8.6, ionic strength 0.075, on

[2] It is not implied the USDA recommends the above company or its product to the possible exclusion of others in the same business.

Whatman paper No. 1. Most of the experiments were done with 175 v., which gave a current flow of about 4.5 ma. Usually, 0.01 ml. of a 6% solution of the substance under test was applied to the apex of the paper. Paper chromatography was carried out with n-butanol acetic acid water pyridine as solvent for 16 to 18 hr. by an ascending technique.

Staining was performed with ninhydrin and bromophenol blue (2).

Soluble products. The hydrolysis of β-casein resulting from the action of rennin or pepsin, measured by the amount of soluble nitrogen and phosphorus with three precipitation conditions (pH 4.7, 2% TCA, 12% TCA) is shown in Table 1. Precipitation with 12% TCA gave a very small soluble fraction,

TABLE 1

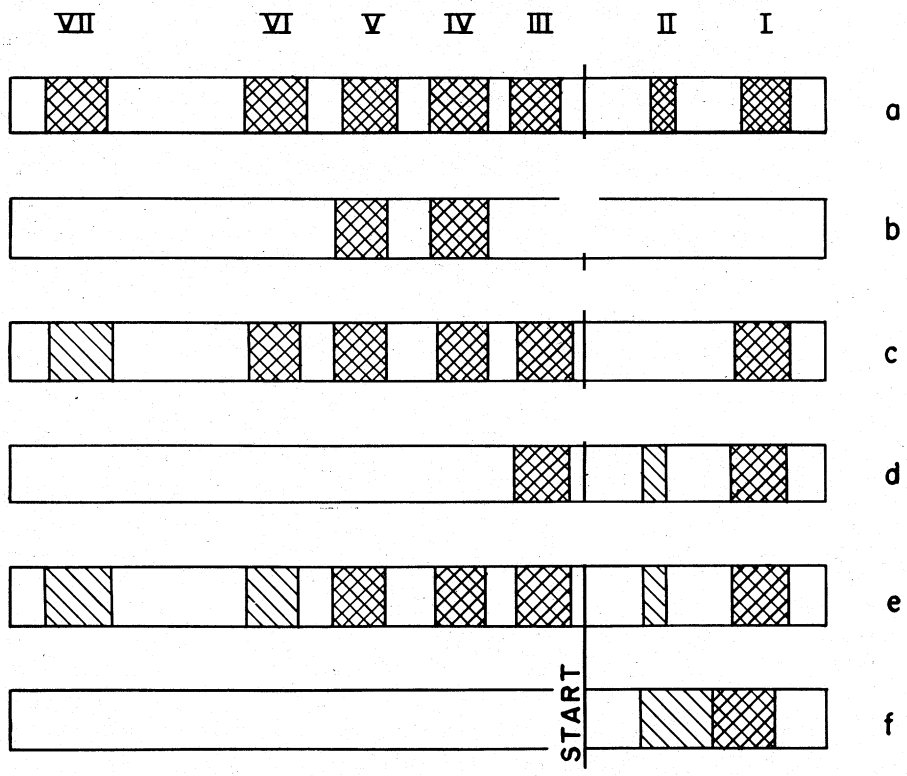
Hydrolysis of β-casein by rennin and pepsin at pH 6.4: soluble nitrogen and phosphorus obtained with three precipitation conditions

Precipitation conditions	Initial soluble	Rennin		Pepsin		
		5 min.	1 hr.	5 min.	1 hr.	5.5 hr.
		Nitrogen (% of total N)				
pH 4.7	0.80	3.46	7.60	3.62	17.49	35.00
2% TCA	0.20	0.92	3.35	1.46	15.81	.....
12% TCA	0.20	0.27	0.98	0.58	2.37	.....
		Phosphorus (% of total P)				
pH 4.7	0.48	2.04	2.39	3.53	2.70	.....
2% TCA	0.15	1.79	1.51	0.77	1.05	.....
12% TCA	0.15	0.85	0.69	0.77	0.77	.....

far less than the amounts obtained by other precipitants. Precipitation at pH 4.7 gave the largest soluble fraction, whereas those experiments with 2% TCA gave somewhat less. Under the conditions used, pepsin hydrolyzed β-casein more extensively than did rennin, particularly with longer periods of time.

The results of paper electrophoretic analysis of pepsin-treated β-casein fractions are shown in Figure 1. The rennin diagram for the pH 4.7–soluble fraction is almost identical with the pepsin diagram shown (a), except that in the Band I region, two very close bands are obtained with rennin. The diagram for the 2% TCA–soluble fraction (not shown) indicates the same number of components as that for the pH 4.7–soluble fraction, but some were reduced in amount. The quantitative relationship between bands shown in Figure 1 was obtained after 5 min. and 1 hr. of proteolysis. The pattern for the pH 4.7–soluble fraction was identical, even when proteolysis was for 5.5 hr. In the pH 4.7–insoluble fraction, however, when proteolysis exceeded 1 hr., the β-casein band was no longer apparent. Bands I and III are the major components of the soluble fractions, with the other cross-hatched bands (Figure 1) intermediate in amount, and the remaining bands relatively minor in amount.

Fractionation of the pH 4.7–soluble material with TCA revealed that the bands corresponding to I, and III to VII, were found in the 2% TCA–insoluble fraction (Figure 1c). All bands in both insoluble fractions gave positive reactions with ninhydrin and bromophenol blue reagents. A qualitative difference between the action of rennin and pepsin was noted in the 12% TCA–insoluble



Schematic representation of paper electrophoretic bands of fractions of β-casein obtained after the action of pepsin for 5 or 60 min. Electrophoresis was at pH 8.6 for 16 hr. (2). Protein bands were evident after staining with bromophenol blue. Cross-hatching is used to indicate the more strongly stained bands. Component IV is β-casein. Components migrating to the left are negatively charged.

FIG. 1. (a) pH 4.7–soluble fraction; (b) pH 4.7–insoluble fraction; (c) pH 4.7–soluble, 2% TCA-insoluble fraction; (d) pH 4.7–soluble, 12% TCA-insoluble fraction; (e) 12% TCA-insoluble, pH 4.7 soluble fraction; (f) 12% TCA-soluble fraction.

fraction (Figure 1d); Band I was the major one for pepsin and Band III was major for rennin. The 12% TCA–soluble fraction was small in amount and contained only one or two bands (Figure 1f). These components gave a positive reaction with ninhydrin only.

β-Casein and all of the insoluble and soluble fractions were studied by paper chromatography. β-Casein and the enzyme-transformed β-casein remained at the origin, with some forward streaking. All other fractions gave spots with greater movement. The results showed that Fractions d and e (See Figure 1) were much more heterogeneous than expected from the electrophoretic results, with movement of 0 to 32 cm. in a 16-hr. run. Further, the major bands (I and III) of e could not be identical with the I and III bands of d, for the number and distribution of the components revealed by chromatography differed greatly

for these two fractions. The 12% TCA–soluble fraction showed six to eight components by chromatography, more than observed by electrophoresis, with movement of 2 to 32 cm. in a 16-hr. run. Differences also were observed between the split products obtained with rennin and pepsin in both the pH 4.7–soluble, 12% TCA–insoluble, and the 12% TCA–soluble fractions.

Insoluble products. The paper electrophoretic pattern of the fraction obtained by precipitation at pH 4.7 is shown in Figure 1b. The 12 and 2% TCA precipitates gave, after reprecipitation at pH 4.7, electrophoretic patterns like Figure 1b. The portion soluble at pH 4.7 obtained by reprecipitation of the 2% TCA–insoluble fraction was heterogeneous as shown by paper electrophoresis (Figure 1c). It contained β-casein (IV), enzyme-transformed β-casein (V), and all the components made soluble by the two enzymes. Both moving boundary and paper electrophoretic studies at pH 8.6 showed that the insoluble fraction was not merely unaltered β-casein, but that a new component differing in electrical charge resulted from the action of rennin and pepsin. This altered casein migrated much faster than β-casein, and slower than α-casein. The concentration of this fraction increased with the reaction time, and in the 5.5 hr. treatment by pepsin all of the original β-casein had been transformed to the new electrophoretic component, which contained 65% of the original β-casein nitrogen (See Table 2). This new electrophoretic fraction was not

TABLE 2

Electrophoretic composition of the insoluble fraction of β-casein (pH 4.7) after hydrolysis with rennin and pepsin

Reaction time with enzymes	pH [a]	Rennin		Pepsin			
		β-casein	Altered β-casein	β-casein	Altered β-casein	Altered β-casein I	Altered β-casein II
		(%)					
5 min.	8.6	92	8	72	28	....	....
1 hr.	8.6	74	26	12	88	....	....
1 hr.	6.7	....	....	11	89	....	....
1 hr.	5.6	....	....	16	84	62	22
5.5 hr.	5.6	....	....	0	100	36	64

[a] pH at which electrophoresis was performed with the moving boundary technique.

homogeneous, for electrophoresis at pH 5.6 revealed two peaks. The ratio of the two peaks was not constant but with time the faster (II) increased at the expense of the slower, as shown in Table 2.

DISCUSSION

The present results comparing the use of several precipitants indicate that the enzymes rennin and pepsin cause a considerable proteolysis of β-casein. This was evident when pH 4.7 or 2% TCA precipitation was used after the action of these enzymes. When 12% TCA was the precipitant, as was the case in the experiments of Nitschmann and collaborators (6, 7), the soluble fraction was negligible. On the basis of similar results, Nitschmann *et al.* (6, 7) had concluded that rennin did not hydrolyze β-casein appreciably, a conclusion that the present results show to be erroneous.

Paper electrophoretic analysis of the soluble fractions showed that the variation in the amount found with the different precipitants was not due merely to difference in degree of solubility of the enzyme-transformed β-casein. There are many more components present in the pH 4.7 soluble fraction than in the 12% TCA–soluble fraction. From this standpoint, the portion soluble at pH 4.7 is the truer measure of hydrolysis.

The amount of proteolysis leading to an increase in the soluble fraction increased with the time of action of the enzymes. The action of pepsin was much more extensive than that of rennin, particularly with longer periods of time. Since the paper electrophoretic patterns of the soluble fraction were essentially the same over a period of 5.5 hr., a general proteolysis probably takes place from the beginning of the reaction. During this same period, free electrophoresis at pH 8.6 shows that the β-casein is transformed to a new electrophoretic component of greater mobility. Electrophoresis at pH 5.6 shows that this fraction is heterogeneous and changes in composition in a regular way with time. Paper electrophoretic patterns and paper chromatography disclosed that the soluble fractions were very complex, containing eight to ten or more components. Some of these components were present only in traces, and it was impossible to give the exact number of peptides.

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